United States Environmental Protection Agency
Office of Water Washington, DC 20460
EPA-821-R-97-004 May 1997

Method 1600: Membrane Filter Test Method for Enterococci In Water

Acknowledgments

This method was developed under the direction of James W. Messer and Alfred P. Dufour of the U.S. Environmental Protection Agency's (EPA) Human Exposure Research Division, National Exposure Research Laboratory, Cincinnati, Ohio. The method document was prepared under EPA Contract 68-C3-0337 by the DynCorp Environmental Programs Division.

Disclaimer

This method has been reviewed and approved for publication by the Office of Science and Technology within EPA's Office of Water. This method is approved for use in ambient water monitoring, but not for wastewater analysis under 40 CFR part 136. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Questions concerning this method or its application should be addressed to:

William A. Telliard, Director
Analytical Methods Staff
Engineering and Analysis Division (4303)
USEPA Office of Water
401 M Street, SW
Washington, DC 20460
Phone: 202-260-7120
Fax: 202-260-7185

Introduction

EPA has been increasingly concerned with the public health risks of infectious diseases caused by microbial organisms in our nation's beaches. To counteract this problem, EPA has established the Beaches Evironmental Assessment Closure and Health (BEACH) Program. This analytical method is published for use in the BEACH Program.

In 1986, EPA issued a revision to its bacteriological ambient water quality criteria recommendations to include new indicator bacteria, *E. coli* and enterococci, which provide a better correlation with swimming-associated gastrointestinal illness than the previous criteria recommendations for fecal coliform bacteria. These revised criteria are useful to public health officials because they enable quantitative estimates of illness rates associated with swimming in polluted water.

This method is a revision of EPA's previous enterococci method, used since 1985 in ambient water quality monitoring. It reduces analysis time to 24 hours and improves analytical quality. The method has been validated in single- and multi-laboratory studies and has undergone peer review.

Requests for additional copies of this method should be directed to:

USEPA National Center for Environmental
Publications and Information (NCEPI)
11029 Kenwood Road
Cincinnati, OH 45242
Phone: 513-489-8190
Document No. EPA-821-R-97-004

Water Resource Center
Mail Code RC- 4100
401 M Street, S.W.
Washington, D.C. 20460
202-260-7786
Document No. EPA-821-R-97-004

Method 1600: Membrane Filter Test Method for Enterococci in Water

1.0 Scope and Application

1.1 This method describes a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. Enterococci are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens.

1.2 The enterococci test measures the bacteriological quality of recreational waters. Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on the established relationship between health effects and water quality. The significance of finding enterococci in recreational water samples is the direct relationship between the density of enterococci in the water and swimming-associated gastroenteritis studies of marine and fresh water bathing beaches (1,2).

1.3 The test for enterococci can be applied to potable, fresh, estuarine, marine, and shellfish growing waters.

1.4 Since a wide range of sample volumes or dilutions can be analyzed by the MF technique, a wide range of enterococci levels in water can be detected and enumerated.

2.0 Summary of Method

2.1 The MF method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter. A water sample is filtered through the membrane which retains the bacteria. Following filtration, the membrane containing the bacterial cells is placed on a selective medium, mEI agar, and incubated for 24 h at 41°C. All colonies with any blue halo are recorded as enterococci colonies, regardless of colony color. Magnification and a small fluorescent lamp are used for counting to give maximum visibility of colonies.

3.0 Definition

3.1 In this method, enterococci are those bacteria which produce colonies with a blue halo after incubation on mEI agar.

4.0 Interferences

4.1 Water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.

5.0 Safety

5.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment.

5.2 Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

6.1 Glass lens with magnification of 2-5X or stereoscopic microscope.

6.2 Lamp, with a cool, white fluorescent tube.

6.3 Hand tally or electronic counting device.

6.4 Pipet container, stainless steel, aluminum or borosilicate glass, for glass pipets.

6.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

6.6 Graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper and sterile.

6.7 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterile.

6.8 Ultraviolet unit for sanitization of the filter funnel between filtrations (optional).

6.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used.

6.10 Flask, filter, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

6.11 Flask for safety trap placed between the filter flask and the vacuum source.

6.12 Forceps, straight or curved, with smooth tips to handle filters without damage.

6.13 Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.

6.14 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles.

6.15 Thermometer, checked against a National Institute of Standards and Technology (NIST) certified thermometer, or one that meets the requirements of NIST Monograph SP 250-23.

6.16 Petri dishes, sterile, plastic, 50 x 12 mm, with tight-fitting lids.

6.17 Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1-100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1-10 dilutions.

6.18 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume.

6.19 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 + 0.02 μm pore size.

6.20 Inoculation loops, at least 3-mm diameter, and needles, nichrome or platinum wire, 26 B & S gauge, in suitable holders.

6.21 Incubator maintained at 41 ±0.5°C.

6.22 Waterbath maintained at 44-46°C for tempering agar.

6.23 Test tubes, 150 x 20 mm, borosilicate glass or plastic.

6.24 Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes.

6.25 Test tubes, screw-cap, borosilicate glass, 125 x 16 mm or other appropriate size.

7.0 Reagents and Standards

7.1 Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (3). The agar used in preparation of culture media must be of microbiological grade.

7.2 Whenever possible, use commercial culture media as a means of quality control.

7.3 Purity of Water: Reagent water conforming to Specification D1193, Reagent water conforming Type II, Annual Book of ASTM Standards (4).

7.4 Buffered Dilution Water

7.4.1 Composition:

Sodium Dihydrogen Phosphate	0.58 g
Sodium Monohydrogen Phosphate	2.50 g
Sodium Chloride	8.50 g

7.4.2 Preparation: Dissolve the ingredients in 1 L of reagent water in a flask and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 lb pressure) for 15 min. The final pH should be 7.4 ± 0.2.

7.5 mEI Agar

7.5.1 Composition of Basal Medium (mE Agar, Difco 0333)

Peptone	10.0 g
Sodium Chloride	15.0 g
Yeast Extract	30.0 g
Esculin	1.0 g
Actidione	0.05 g
Sodium Azide	0.15 g
Agar	15.0 g

7.5.2 Preparation of mEI medium: Add 71.2 g of dehydrated basal medium plus 0.75 grams of indoxyl B-D glucoside to 1 L of reagent grade water in a flask and heat to boiling until ingredients dissolve. Autoclave at 121°C and 15 lb pressure for 15 min and cool in a 44-46°C water bath.

7.5.3 Reagents added after sterilization: Mix 0.24 g nalidixic acid in 5 mL reagent grade water, add a few drops of 0.1N NaOH to dissolve; add to the mEI medium. Add 0.02 g triphenyl tetrazolium chloride separately to the mEI medium and mix.

7.5.4 Preparation of mEI Agar Plates: Pour the mEI agar into 50 mm petri dishes to a 4-5 mm depth (approximately 4-6 mL), and allow to solidify. The final pH of medium should be 7.1 ± 0.2. Store in a refrigerator.

7.6 Brain Heart Infusion (BHI) (Difco 0037-02, BBL 11058)

7.6.1 Composition:

Calf Brain Infusion	200.0 g
Beef Heart Infusion	250.0 g
Peptone	10.0 g
Sodium Chloride	5.0 g
Disodium Phosphate	2.5 g
Dextrose	2.0 g

7.6.2 Preparation: Dissolve 37 g of dehydrated brain heart infusion in 1 L of reagent grade water. Dispense in 8-10 mL volumes in screw-cap tubes and autoclave at 121°C (15 lb pressure) for 15 min. If the medium is not used the same day as prepared and sterilized, heat in boiling water bath for several min to remove absorbed oxygen, and cool quickly without agitation, just prior to inoculation. The final pH should be 7.4 ± 0.2.

7.7 Brain Heart Infusion (BHI) Broth with 6.5% NaC1

7.7.1 Composition: Brain heart infusion broth with 6.5% NaC1 is the same as BHI broth in 7.6 with additional NaC1.

7.7.2 Preparation: Add 60.0 g NaC1 per liter of medium. Since most commercially available dehydrated media contain sodium chloride, this amount is taken into consideration in determining the final NaCl percentage above.

7.8 Brain Heart Infusion Agar (Difco 0418-02, BBL 11064)

7.8.1 Composition: Brain heart infusion agar contains the same components as BHI (see 7.6) with the addition of 15.0 g of agar per L of BHI broth.

7.8.2 Preparation: Heat to boiling until ingredients are dissolved. Dispense 10-12 mL of medium in screw-cap test tubes and sterilize for 15 min at 121°C (15 lb pressure). Slant after sterilization. The final pH should be 7.4 ± 0.2.

7.9 Bile Esculin Agar (BEA) (Difco 0879)

7.9.1 Composition:

Bacto Beef Extract	3.0 g
Bacto Peptone	5.0 g
Bacto Oxgall	40.0 g
Bacto Esculin	1.0 g
Ferric Citrate	0.5 g
Bacto Agar	15.0 g

7.9.2 Preparation: Add 64.5 g of dehydrated BEA to 1 L reagent water and heat to boiling to dissolve completely. Dispense in 8-10 mL volumes in tubes for slants or into flasks for subsequent plating. Autoclave at 121°C at 15 lb pressure for 15 min. Overheating may cause darkening of the medium. Cool to 44-46°C and dispense into sterile petri dishes. The final pH should be 6.6 ± 0.2. Store in a refrigerator.

8.0 Sample Collection, Preservation, and Storage

8.1 Sampling procedures are described in detail in the USEPA microbiology methods manual, Section II, A (5). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples shall not be analyzed if these conditions are not met.

8.1.1 Storage Temperature and Handling Conditions

Ice or refrigerate bacteriological samples at a temperature of 1-4°C during transit to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

8.1.2 Holding Time Limitations

Examine samples as soon as possible after collection. Do not hold samples longer than 8 h between collection and initiation of analyses.

9.0 Quality Control

9.1 See recommendations on quality control for microbiological analyses in the USEPA microbiology methods manual, Part IV, C (5).

10.0 Calibration and Standardization

10.1 Check temperatures in incubators daily to ensure operation within stated limits.

10.2 Check thermometers at least annually against an NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250-23. Check mercury columns for breaks.

11.0 Procedure

11.1 Prepare the mEI agar as directed in 7.5.

11.2 Mark the petri dishes and report forms with sample identification and sample volumes.

11.3 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base; the membrane filter is now held between the funnel and the base.

11.4 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel.

11.5 For ambient surface waters and wastewaters, select sample volumes based on previous knowledge of the pollution level, to produce 20-60 enterococci colonies on membranes. Sample volumes of 1-100 mL are normally tested at half log intervals, for example 100, 30, 10, 3 mL, etc.

11.6 Smaller sample size or sample dilution can be used to minimize the interference of turbidity or high bacterial densities. Multiple volumes of the same sample or dilution of sample may be filtered and the results combined.

11.7 Filter the sample and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum and remove the funnel from the filter base.

11.8 Use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the mEI agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Close the dish, invert, and incubate at 41 ± 0.5°C for 24 h.

11.9 After incubation, count and record colonies on those membrane filters containing, if practical, 20-60 colonies with any blue halo regardless of colony color as an enterococci colony. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

12.0 Data Analysis and Calculations

Use the following general rules to calculate the enterococci count per 100 mL of sample:

12.1 Select and count membranes with ideally 20-60 colonies with any blue halo as an enterococci colony. Calculate the final value using the formula:

$$\textit{Enterococci/100mL} = \frac{\textit{No. of enterococci colonies}}{\textit{Volume of sample filtered(mL)}} \times 100$$

12.2 See the USEPA microbiology manual, Part II, Section C, 3.5, for general counting rules (5).

13.0 Method Performance

13.1 Specificity - The specificity of the medium used in this method is 6.0% false positive and 6.5% false negative for various environmental water samples (6). The false positive rate was calculated as the percent of colonies which reacted typically, but did not verify as members of the enterococcus group. The false negative rate was calculated as the percent of all verified enterococcus colonies not reacting typically.

13.2 Bias - The persistent positive or negative deviation of the results from the assumed or accepted true value is not significant (6).

13.3 Precision - The precision among laboratories for marine water and surface water was 2.2% and 18.9% (6).

14.0 Reporting Results

14.1 Report the results as enterococci per 100 mL of sample.

15.0 Verification Procedure

15.1 Colonies with any blue halo can be verified as enterococci. Verification of colonies may be required in evidence gathering, and is also recommended as a QC procedure upon initial use of the test and with changes in sample sites or lots of commercial media. The verification procedure follows.

15.2 Using a sterile inoculating needle, transfer cells from the centers of at least 10 well-isolated typical colonies into a brain heart infusion broth (BHI) tube and onto a BHI slant. Incubate broth tubes for 24 h and slants for 48 h at 35 ± 0.5°C.

15.3 After 24 h incubation, transfer a loopful of material from each BHI broth tube to:

Bile Esculin Agar (BEA) and incubate at 35± 0.5°C for 48 h.
BHI Broth and incubate at 45 ± 0.5°C for 48 h.
BHI Broth with 6.5% NaC1 and incubate at 35 ± 0.5°C for 48 h.

15.4 Observe for growth.

15.5 After 48 h incubation, apply a gram stain to growth from each BHI agar slant.

15.6 Gram positive cocci which grow in BEA, BHI Broth at 45°C, and BHI Broth + 6.5% NaCl, and hydrolyze esculin, are verified as enterococci.

16.0 Pollution Prevention

16.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.

16.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

17.0 Waste Management

17.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.

17.2 Samples, reference materials, and equipment known or suspected to have viable enterococci attached or contained must be sterilized prior to disposal.

17.3 Samples preserved with HCl to pH <2 are hazardous and must be neutralized before being disposed, or must be handled as hazardous waste.

17.4 For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" and "Less Is Better: Laboratory Chemical Management for Waste Reduction," both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street N.W., Washington, D.C. 20036.

18.0 References

18.1 Cabelli, V. J. 1980. Health Effects Criteria for Marine Recreational Waters, EPA-600/1-80-031. Office of Research and Development, USEPA.

18.2 Dufour, A.P. 1984. Health Effects Criteria for Fresh Recreational Waters, EPA-600/1-84-004. Office of Research and Development, USEPA.

18.3 Reagent Chemicals, American Chemical Society Specifications, American Chemical Society, Washington, DC. For suggestions of the testing of reagents not listed by the American Chemical Society, see Analar Standards for Laboratory Chemicals, BDH Ltd., Poole, Dorset, UK and the United States Pharmacopeia.

18.4 Annual Book of ASTM Standards, Vol. 11.01, American Society for Testing and Materials, Philadelphia, PA 19103.

18.5 Bordner, R., J.A. Winter and P.V. Scarpino (eds.), Microbiological Methods for Monitoring the Environment, Water and Wastes, EPA-600/8-78-017. Office of Research and Development, USEPA.

18.6 Messer, J.W. and A.P. Dufour. 1997. Personal Communication. USEPA.